AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Amino Acid Substitutions Account for Most MexS Alterations in Clinical *nfxC* Mutants of *Pseudomonas aeruginosa*

Charlotte Richardot,[a] Paulo Juarez,[a] Katy Jeannot,[a,b] Isabelle Patry,[b] Patrick Plésiat,[a,b] Catherine Llanes[a]

Laboratoire de Bactériologie EA4266, Faculté de Médecine-Pharmacie, Université de Franche-Comté, Besançon, France[a]; Laboratoire de Bactériologie, Centre Hospitalier Universitaire de Besançon, Besançon, France[b]

Multidrug-resistant mutants of *Pseudomonas aeruginosa* that overproduce the active efflux system MexEF-OprN (called *nfxC* mutants) have rarely been characterized in the hospital setting. Screening of 221 clinical strains exhibiting a reduced susceptibility to ciprofloxacin (a substrate of MexEF-OprN) and imipenem (a substrate of the negatively coregulated porin OprD) led to the identification of 43 (19.5%) *nfxC* mutants. Subsequent analysis of 22 nonredundant mutants showed that, in contrast to their *in vitro*-selected counterparts, only 3 of them (13.6%) harbored a disrupted *mexS* gene, which codes for the oxidoreductase MexS, whose inactivation is known to activate the *mexEF-oprN* operon through a LysR-type regulator, MexT. Nine (40.9%) of the clinical *nfxC* mutants contained single amino acid mutations in MexS, and these were associated with moderate effects on resistance and virulence factor production in 8/9 strains. Finally, the remaining 10 (45.5%) *nfxC* mutants did not display mutations in any of the regulators known to control *mexEF-oprN* expression (the *mexS*, *mexT*, *mvaT*, and *ampR* genes), confirming that other loci are responsible for pump upregulation in patients. Collectively, these data demonstrate that *nfxC* mutants are probably more frequent in the hospital than previously thought and have genetic and phenotypic features somewhat different from those of *in vitro*-selected mutants.

Pseudomonas aeruginosa is a notorious cause of acute and chronic infections in vulnerable patients. The ability of this environmental Gram-negative bacterium to produce a broad range of virulence factors (1) and to become resistant to multiple antimicrobial agents is considered a key to its success in the hospital setting. When overexpressed upon mutation, several efflux systems belonging to the resistance-nodulation-cell division (RND) family of drug transporters are able to decrease the susceptibility of the pathogen to structurally unrelated antibiotics (2). One of these systems, named MexEF-OprN, is quiescent in wild-type strains grown under standard laboratory conditions. Its contribution to the intrinsic resistance of *P. aeruginosa* is therefore minimal. In contrast, in so-called *nfxC* mutants, stable overproduction of the pump results in a significant increase in the MICs (4- to 16-fold) of chloramphenicol, trimethoprim, and fluoroquinolones (3). Compared with the susceptibility of wild-type strains, typical *nfxC* mutants exhibit a hypersusceptibility to some antipseudomonal β-lactams (penicillins, cephalosporins) and aminoglycosides, a phenotype possibly due to the impaired activity of two other RND pumps, namely, MexAB-OprM and MexXY/OprM (4). Furthermore, this typical NfxC phenotype includes a decreased susceptibility to carbapenems, linked to the downregulation of the *oprD* gene, which codes for the specific porin OprD, allowing the facilitated diffusion of these antibiotics into the cell (3).

In *P. aeruginosa*, while most RND pumps have their expression modulated by repressors (5), transcription of the *mexEF-oprN* operon is controlled by a LysR-type activator, MexT, encoded by an adjacent gene (6). In some drug-susceptible laboratory strains of the PAO1 lineage, *mexT* is inactivated by an 8-bp insert (7). Spontaneous excision of this intragenic fragment restores the open reading frame of *mexT* with the concomitant overexpression of *mexEF-oprN* and the development of the typical NfxC phenotype (6). In other strains, *mexEF-oprN* transcription is triggered by mutations in another gene, *mexS*, which is divergently transcribed from *mexT* and encodes an oxidoreductase (8). In any case, a functional MexT is mandatory for the *in vitro* selection of MexEF-OprN-overproducing mutants. This regulator has been reported to increase *mexS* expression (6), even if the consensus *nod*-box DNA sequence, considered the binding site of MexT, remains to be identified in the promoter region of *mexS* (9). To explain the MexS/MexT-dependent regulation of *mexEF-oprN*, it has been postulated that MexS is involved in the detoxification of some endogenously produced MexT-activating molecule(s) (10, 11). In this scenario, if it is not processed by MexS, the toxic metabolite(s) would be exported out of the cell by MexEF-OprN as a rescue mechanism.

In clinical strains, *nfxC* mutations are difficult to characterize because of polymorphic variations in the MexS and MexT protein sequences (http://pseudomonas.com). Moreover, data suggest that still uncharacterized pathways might influence *mexEF-oprN* expression (12). Supporting this notion, *in vitro* mutants with alterations in the *mvaT*, *ampR*, or *mxtR* gene have been reported to overexpress *mexEF-oprN* and to exhibit a multidrug resistance phenotype (13–15). However, the relevance of such mutations in clinical strains awaits confirmation.

In vitro-selected *nfxC* mutants were found to be deficient in the

Received 29 October 2015 **Returned for modification** 13 November 2015
Accepted 25 January 2016

Accepted manuscript posted online 1 February 2016

Citation Richardot C, Juarez P, Jeannot K, Patry I, Plésiat P, Llanes C. 2016. Amino acid substitutions account for most MexS alterations in clinical *nfxC* mutants of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 60:2302–2310. doi:10.1128/AAC.02622-15.

Address correspondence to Catherine Llanes, cllanesb@univ-fcomte.fr.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02622-15.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Pseudomonas aeruginosa		
PAO1	Wild-type reference strain PAO1-UW (University of Washington)	B. Holloway
PAO7H	*nfxC* mutant derived from wild-type strain PAO1-UW	3
PA14	Wild-type reference strain PA14	B. Ausubel
PA14ΔS	PA14 with a *mexS* (*nfxC*-type) deletion	This study
PA14ΔT	PA14 with a *mexT* deletion	This study
PA14ΔS_{PA14}	PA14 Δ*mexS trans*-complemented with *mexS* from reference strain PA14	This study
PA14ΔS_{PAO1}	PA14 Δ*mexS trans*-complemented with *mexS* from reference strain PAO1	This study
PA14ΔS_{1307}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 1307	This study
PA14ΔS_{2310}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 2310	This study
PA14ΔS_{2505}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 2505	This study
PA14ΔS_{3005}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 3005	This study
PA14ΔS_{0911}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 0911	This study
PA14ΔS_{1009}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 1009	This study
PA14ΔS_{0801}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 0801	This study
PA14ΔS_{1409}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 1409	This study
PA14ΔS_{2311}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 2311	This study
PA14ΔS_{2609}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 2609	This study
PA14ΔS_{1709}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 1709	This study
PA14ΔS_{1711}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 1711	This study
PA14ΔS_{0607}	PA14 Δ*mexS trans*-complemented with *mexS* from clinical strain 0607	This study
PA14ΔT_{0810}	PA14 Δ*mexT trans*-complemented with *mexT* from clinical strain 0810	This study
PA14ΔT_{1510}	PA14 Δ*mexT trans*-complemented with *mexT* from clinical strain 1510	This study
Escherichia coli		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	43
CC118λ*pir*	CC118 lysogenized with λ*pir* phage	44
DH5α	F^- *supE44 endA1 hsdR17*(r_K^- m_K^-) *thi-1 recA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*ΔM15 *phoA gyrA96 relA1 deoR* λ^-	Invitrogen
HB101	*supE44 hsd*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	45
Plasmids		
pCR-Blunt	Cloning vector for blunt-end PCR products, *lacZ*ΔColE1 f1 *ori* Ap^r Km^r	Invitrogen
pRK2013	Helper plasmid, ColE1 *ori* Tra^+ Mob^+ Km^r	25
mini-CTX1	Self-proficient integration vector, *tet* Ω-FRT-*attP*-MCS *ori int oriT* Tc^r[a]	33
pKNG101	Suicide vector in *P. aeruginosa*, *sacB* Sm^r	32

[a] FRT, FLP recombination target; MCS, multiple-cloning site.

production of several quorum-sensing-dependent virulence factors (16) without an apparent loss of fitness (17). The mutants derived from reference strain PAO1 typically produce less pyocyanin, rhamnolipids, and elastase than the wild-type parents (3, 16) and less type III secretion system (T3SS) effector toxin ExoS (18). This phenotype was attributed to (i) reduced intracellular levels of the *Pseudomonas* quinolone signal (PQS), caused by a shortage of a metabolic precursor (kynurenine or 4-hydroxy-2-heptylquinoline [HHQ]) exported by the pump (17, 19), and (ii) MexT acting as a global regulator and indirectly impairing the T3SS in an MexEF-OprN-independent way (18).

Information about the rates and traits of *nfxC* mutants in cystic fibrosis (CF) patients (20, 21) and non-CF patients (12, 22–24) remains scarce. As a plausible explanation, the low virulence of these mutants would be detrimental to their survival in the host or in the hospital setting and would account for their infrequent isolation from clinical samples. Alternatively, these mutants would be phenotypically and genetically distinct from their *in vitro* counterparts (i.e., they would keep some degree of pathogenicity or persistence) and thus would be underrecognized. In this study, we show that most clinical *nfxC* mutants have mild defects in MexS and are still able to produce substantial amounts of virulence factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The reference strains and cloning plasmids used in this study are listed in Table 1. Twenty-two clinical *nfxC* mutants collected between May 2012 and May 2013 at the University Hospital of Besançon, Besançon, France (see Table S1 in the supplemental material), and 7 drug-susceptible strains of *P. aeruginosa* collected from surface waters (PE1, PE1346, PE1361, PE1393, PE1423, PE1446, and PE1450) were also investigated. All the bacterial cultures were grown in Mueller-Hinton broth (MHB) with adjusted concentrations of Ca^{2+} (range, 20 to 25 mg $liter^{-1}$) and Mg^{2+} (range, 10 to 12.5 mg $liter^{-1}$) (Becton Dickinson and Company, Cockeysville, MD) or on Mueller-Hinton agar (MHA; Bio-Rad, Marnes-la-Coquette, France). *Escherichia coli* transformants were selected on MHA containing 50 μg ml^{-1} kanamycin (a marker of the vector pCR-Blunt), 15 μg ml^{-1} tetracycline (a marker of the vector mini-CTX1), or 50 μg ml^{-1} streptomycin (a marker of the vector pKNG101). Recombinant plasmids were introduced into *P. aeruginosa* strains by triparental matings and mobilization with broad-host-range vector pRK2013 in *E. coli* HB101 as a helper strain (25). Transconjugants were selected on *Pseudomonas* isolation agar (PIA;

Becton, Dickinson and Company) supplemented with 200 μg ml^{-1} tetracycline or 2,000 μg ml^{-1} streptomycin, as required. Excision of pKNG101 was obtained by selection on M9 minimal medium (8.54 mM NaCl, 25.18 mM NaH_2PO_4, 18.68 mM NH_4Cl, 22 mM KH_2PO_4, 2 mM $MgSO_4$, pH 7.4) supplemented with 5% sucrose and 0.8% agar.

Antibiotic susceptibility testing. The MICs of selected antibiotics were determined by the standard serial 2-fold dilution method in MHA with an inoculum of 10^4 CFU per spot, according to CLSI recommendations (26). Growth was assessed visually after 18 h of incubation at 37°C.

RT-qPCR experiments. Specific gene expression levels were measured by real-time quantitative PCR (RT-qPCR) after reverse transcription, as described previously (27). Briefly, 2 μg of total RNA was reverse transcribed with ImProm-II reverse transcriptase as specified by the manufacturer (Promega, Madison, WI). The amounts of specific cDNA were assessed in a Rotor Gene RG6000 instrument (Qiagen, Courtaboeuf, France) by using a QuantiFast SYBR PCR green kit (Qiagen). When primers were not already published, the primers used for amplification were designed from the gene sequences available in the *Pseudomonas* Genome Database, version 2, by using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (see Table S2 in the supplemental material). For each strain, the mRNA levels of the target genes were normalized to those of the *rpsL* housekeeping gene and expressed as a ratio to the level in wild-type reference strain PA14. Mean gene expression values were calculated from two independent bacterial cultures, each of which was assayed in duplicate. Strain PA14ΔS was used as a positive control for *mexE* gene overexpression. As shown in preliminary experiments, all *mexE* transcript levels ≥20-fold above the *mexE* transcript level of PA14 were associated with a decreased susceptibility (≥2-fold) of the strains to MexEF-OprN substrate antibiotics and considered significant.

Virulence factor analysis. Biofilm production was assessed by measuring bacterial adhesion to 96-well polystyrene plates (28). Cultures were incubated in triplicate in MHB medium overnight at 30°C and washed twice with 200 μl of distilled water to eliminate planktonic bacteria. Attached bacteria were colored by 1% (wt/vol) crystal violet and solubilized by 99% (vol/vol) ethanol. Attachment was evaluated at 600 nm and considered negative when the optical density (OD) was <1, as previously reported (19).

Swarming motility was tested on freshly prepared M8 medium (42.2 mM Na_2HPO_4, 22 mM KH_2PO_4, 7.8 mM NaCl, pH 7.4) supplemented with 2 mM $MgSO_4$, 0.5% casein, 0.5% agar, and 1% glucose (29). After 15 min of incubation of the plates at 37°C, 5 μl of culture (7.5×10^5 CFU) was spotted onto the medium surface, with strain PA14 being used as a positive control. The formation of dendrites after 24 h of culture at 37°C was considered a positive result, while a steady spot was considered negative.

Elastase activity was assessed by using MHA plates supplemented with 4 mg ml^{-1} elastin-Congo red (Sigma-Aldrich, St. Louis, MO) and inoculated with 5-μl volumes of bacterial suspension (7.5×10^5 CFU). Enzymatic degradation of the substrate formed clear halos around the culture spots after 48 h of incubation at 37°C (30). The absence of a visible halo was considered a negative result.

Rhamnolipid production was appreciated using a hemolysis assay. Briefly, after 18 h of growth at 37°C in agitated MHB ($A_{600} = 6.7 \pm 0.4$), bacterial supernatants containing rhamnolipids were collected and mixed with defibrinated horse blood diluted 1/100 (vol/vol) in phosphate-buffered saline. After 30 min of incubation at room temperature, the mixture was centrifuged for 10 min at 950 × *g*. The concentration of hemoglobin in the supernatants was determined spectrophotometrically at 405 nm. OD values were expressed as the percent hemolysis relative to the complete hemolysis achieved with Triton X-100 (by definition, 100%). The results presented are mean values from two independent experiments. Hemolytic activity was considered to be significantly reduced when it was less than 50% of that for the control.

Finally, pyocyanin assays were carried out on culture supernatants after 18 h of growth at 37°C in a specific broth [120 mM Tris HCl, pH 7.2, 0.1% tryptone, 20 mM $(NH_4)_2SO_4$, 1.6 mM $CaCl_2$, 10 mM KCl, 24 mM sodium citrate, 50 mM glucose] ($A_{600} = 1.6 \pm 0.2$). The pigment was extracted from the cultures with chloroform (1 volume) and mixed with 0.1 M HCl (0.06 volume) before spectrophotometric measurement at 520 nm (31). Results are mean values from two independent experiments. Pyocyanin production was considered to be significantly reduced when it was less than 50% of that of reference strain PA14 grown under the same conditions.

The virulence factor production of individual clinical strains was rated by a global score ranging from 0 to 5, which corresponds to the number of positive or significant results obtained by each of the 5 assays mentioned above, the result of each of which was given a value of 1 if it was positive or significant.

Construction of deletion mutants from strain PA14. Single *mexS* and *mexT* deletion mutants were constructed by using overlapping PCRs and recombination events, as described by Kaniga et al. (32). First, the 5′ and 3′ regions flanking *mexS* (417 and 433 bp, respectively) and *mexT* (408 and 453 bp, respectively) were individually amplified by PCR with specific primers (see Table S2 in the supplemental material) under the following conditions: 3 min of denaturation at 98°C followed by 30 cycles of amplification, each of which was composed of 10 s at 98°C, 30 s at 60°C, and 30 s at 72°C, and a final extension step of 7 min at 72°C. The resultant amplicons were used as the templates for overlapping PCRs with external pairs of primers to generate the mutagenic DNA fragments. The reaction mixtures contained 1× iProof HF master mix, 3% dimethyl sulfoxide, and 0.5 μM each primer (Bio-Rad). The amplified products were cloned into plasmid pCR-Blunt according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and next subcloned as BamHI/ApaI fragments into the suicide vector pKNG101 in *E. coli* CC118λ*pir* (32). The recombinant plasmids were transferred into *P. aeruginosa* by conjugation and selected on PIA containing 2,000 μg ml^{-1} streptomycin. The excision of the undesired pKNG101 sequence was performed by plating transformants on M9 minimal medium plates containing 5% (wt/vol) sucrose and 1% (wt/vol) glucose. Negative selection on streptomycin was carried out to confirm the loss of the plasmid. The allelic exchanges were confirmed by PCR. Nucleotide sequencing experiments confirmed deletion of 826 bp in *mexS* and 929 bp in *mexT*, yielding strains PA14ΔS and PA14ΔT, respectively.

Chromosomal complementation with full-length *mexS* and *mexT*. A search for mutations in the *mexS* and *mexT* genes, as well as in the *mexS-mexT* and *mexT-mexE* intergenic regions, was performed with 43 clinical strains by using the primers listed in Table S2 in the supplemental material. The mutated *mexS* and *mexT* genes along with their respective promoter regions were amplified from purified genomic DNA by PCR. The resulting DNA fragments were cloned into plasmid pCR-Blunt and next ligated to BamHI/HindIII-linearized plasmid mini-CTX1 (33). The recombinant plasmids were then transferred from *E. coli* CC118 to *P. aeruginosa* strains PA14Δ*mexS* or PA14Δ*mexT* by conjugation with subsequent selection on PIA plates containing 200 μg ml^{-1} tetracycline, to allow their chromosomal insertion into the *attB* site. Chromosomal integration was confirmed by PCR and sequencing.

RESULTS AND DISCUSSION

Wild-type genes *mexS* and *mexT*. Strains PAO1 (3, 8, 34), PA14 (10), and PAK (35) have alternatively been used as wild-type reference strains in studies on the MexEF-OprN efflux pump. However, the *mexS* and *mexT* genes in these strains show a nonsilent sequence polymorphism whose impact on the functionality of the encoded proteins, MexS and MexT, respectively, remained to be clarified. For instance, in addition to carrying an 8-bp intragenic fragment inactivating *mexT* (7), most of the laboratory strains of the PAO1 lineage differ from PA14 (or PAK) by an aspartic acid residue (D) instead of an asparagine (N) at position 249 (D_{249}) in MexS (http://pseudomonas.com).

TABLE 2 Genotypes and resistance profiles of *nfxC* mutants

Strain	Sequence		*mexE* transcript level[c]	MIC (μg ml^{-1})[d]				
	MexS or *mexS*[a]	MexT or *mexT*[b]		CHL	CIP	IMP	TIC	AMK
Reference strains								
PA14	WT	WT	1	64	0.12	1	16	2
PA14ΔS	Δ809 bp (bp 1–809)	WT	**427**	2,048	2	2	8	0.5
PA14ΔS$_{PA14}$	WT	WT	1.9	64	0.12	1	16	2
PA14ΔS$_{PAO1}$	$N_{249}D$	WT	**87**	2,048	2	2	8	1
PA14ΔT	WT	Δ883 bp (bp 32–915)	0.4	64	0.12	1	16	2
PA14ΔT$_{PA14}$	WT	WT	6.2	64	0.12	1	16	2
PAO1	$N_{249}D$	+8 bp (at bp 118)	0.2	16	0.12	1	16	8
PAO7H	$N_{249}D$	WT	**265**	2,048	2	4	8	4
Clinical strains with no mutation in *mexS* and *mexT*								
2502	WT	WT	**35**	256	0.25	8	64	2
1206	WT	WT	**41**	512	0.5	2	64	4
0708	WT	WT	**53**	256	0.25	1	64	4
0309	WT	WT	**28**	128	1	1	64	2
2607	WT	WT	**39**	256	0.5	2	64	4
0712	WT	WT	**325**	2,048	2	4	16	16
0608	WT	WT	**25**	256	0.25	2	64	8
Clinical strains with mutations in *mexS*								
1307	$V_{104}A$	WT	**29**	256	16	2	64	8
2310	$F_{253}L$	WT	**183**	1,024	0.5	4	32	8
2505	$D_{44}E$	WT	**212**	512	64	16	32	4
3005	$S_{60}F$	WT	**259**	2,048	1	4	8	4
0911	$F_{185}L$	WT	**133**	1,024	0.5	4	8	2
1009	$V_{73}A + L_{270}Q$	WT	**312**	1,024	32	8	128	8
0801	$C_{245}G$	WT	**81**	256	0.5	4	128	64
1409	$A_{166}P$	WT	**179**	1,024	2	4	8	2
2311	$S_{60}P$	WT	**455**	1,024	1	4	4	2
2609	$L_{263}Q$	WT	**534**	2,048	1	4	8	2
1709	Δ8 bp (bp 710–718)	WT	**552**	2,048	2	4	8	2
1711	ΔC_{293}	WT	**825**	32	1	2	32	8
0607	Δ30 bp (bp 927–956)	WT	**556**	512	1	8	4	2
Clinical strains with mutations in *mexT*								
0810	WT	$G_{258}D$	**254**	512	8	4	128	2
1510	WT	$Y_{138}D + G_{258}D$	**20**	256	0.25	16	128	1

[a] MexS (339 aa) of PA14 is functional (N_{249}) and is considered the wild type (WT), contrary to PAO1-UW (D_{249}) (www.pseudomonas.com). aa, amino acid.
[b] MexT (304 aa) of PA14 is functional and is considered the wild type, contrary to PAO1-UW (+8 bp [starting at bp 118]) (www.pseudomonas.com).
[c] Expressed as a ratio to that of wild-type reference strain PA14. *nfxC* mutants (the values for which are in bold) have a transcript level of ≥20.
[d] CHL, chloramphenicol; CIP, ciprofloxacin; IMP, imipenem; TIC, ticarcillin; AMK, amikacin.

Because the MexS-D_{249} protein was considered either functional (7) or nonfunctional (35), we deleted *mexS* in both PA14 and PAO1 and compared the effects of this deletion on *mexEF-oprN* expression and antibiotic resistance. In PA14, suppression of *mexS* (strain PA14ΔS) resulted in a strong increase in *mexE* transcription (427-fold) and in a 16- to 32-fold higher resistance to MexEF-OprN substrates, as in typical *nfxC* mutants (Table 2). As expected, complementation of PA14ΔS with the PA14 *mexS* allele (PA14ΔS$_{PA14}$) restored the drug-susceptible phenotype. In contrast, the PAO1 *mexS* allele had virtually no impact on the resistance levels of strain PA14ΔS (strain PA14ΔS$_{PAO1}$) and failed to reverse the overexpression of *mexE*, whose transcripts remained 45-fold more abundant in PA14ΔS in comparison with PA14 *mexS* allele. Consistent with PAO1 producing inactive MexS-D_{249} and MexT peptides, spontaneous excision of the extra 8-bp sequence inserted in *mexT* is known to trigger MexEF-OprN production in this strain, with MexT recovering its functionality in a nonfunctional MexS background (36). Confirming that MexS-N_{249} (and not MexS-D_{249}) is functional, analysis of 7 drug-susceptible strains of *P. aeruginosa* collected from surface waters (PE1, PE1346, PE1361, PE1393, PE1423, PE1446, and PE1450) showed that the genomes of all of them encoded MexS-N_{249} together with an active MexT (without any insertion in the *mexT* gene) (data not shown). Based on these results, we therefore used strain PA14 instead of PAO1 in further experiments to investigate the functionality of MexS and MexT from clinical *nfxC* mutants.

Selection of clinical *nfxC* mutants. We screened a collection of 221 clinical isolates of *P. aeruginosa* exhibiting a reduced suscep-

tibility to ciprofloxacin, a substrate of MexEF-OprN, and imipenem, a substrate of porin OprD, whose expression is inversely coregulated with that of MexEF-OprN (6). The ciprofloxacin and imipenem concentrations used in the screening were equal to the MIC values for reference strain PA14 (0.12 μg ml^{-1} and 1 μg ml^{-1}, respectively; Table 2). As resistance to these antibiotics may also be due to other efflux pumps (e.g., MexXY/OprM, MexCD-OprJ, MexAB-OprM) as well as other mechanisms (e.g., fluoroquinolone target alterations, mutational loss of porin OprD), the levels of the *mexE* transcripts were determined in all the strains by RT-qPCR. Forty-three (19.5%) of the 221 selected isolates were found to significantly overexpress *mexE* (≥20-fold) compared with the level of *mexE* expression by PA14 (data not shown). According to available clinical data, these 43 *nfxC* mutants were involved in the colonization or infection of 17 patients (from 1 to 12 isolates per patient) admitted to various medical and surgical units of University Hospital of Besançon, Besançon, France (see Table S1 in the supplemental material). Most of these patients (12/17) were treated with antibiotics prior to the isolation of the *nfxC* mutant strains, including 7/12 treated with fluoroquinolones known to easily select *nfxC* mutants (22, 37). Sequencing of the *mexS* and *mexT* genes (data not shown) allowed us to identify the redundant mutants in individual patients and to eventually retain 22 strains (1, 2, or 3 different strains per patient) for further investigations (see Table S1 in the supplemental material).

Drug susceptibility of clinical *nfxC* mutant isolates. The level of overexpression of the *mexE* gene was found to vary greatly among the 22 clinical mutants (from 20- to 825-fold the level of expression by PA14; Table 2). These elevated values were associated with an increased resistance of the strains (except strain 1711) to the MexEF-OprN substrates chloramphenicol (MIC range, 2- to 32-fold the MIC for PA14) and ciprofloxacin (MIC range, 2- to 512-fold the MIC for PA14), though the possibility that additional mechanisms may have influenced the drug MICs cannot be excluded. For unclear reasons, one strain, 1711, turned out to be more susceptible (2-fold) to chloramphenicol than PA14, despite the strong upregulation of its *mexE* gene (825-fold). As indicated in Table 2, 20/22 strains exhibited a 2- to 16-fold decrease in susceptibility to imipenem compared with that of PA14, possibly due to the MexT-dependent downregulation of specific porin OprD (6), a mutational loss of OprD, and/or carbapenemase production (38). Finally, the reported hypersusceptibility of typical *in vitro* *nfxC* mutants to the MexAB-OprM substrate ticarcillin and to the MexXY(OprM) substrate amikacin (4) was observed in only 7 strains and 1 strain, respectively, suggesting that this hypersusceptible phenotype either arises in specific genetic backgrounds, such as the PAO1 and PA14 backgrounds, or is masked in most clinical *nfxC* strains by additional mechanisms. It should be noted that because of this phenotypic variability, MexEF-OprN-overproducing mutants may be difficult to recognize in the medical laboratory unless molecular biology techniques are used.

Amino acid variations in the MexT regulator. MexT needs to be functional to activate *mexEF-oprN* operon expression in *nfxC* mutants (6). Concordant with this, DNA sequencing revealed that 20/22 strains (91%) produced a MexT protein identical to that of PA14 (Table 3). Interestingly, 2/22 strains (9%) harbored *mexT* genes with point mutations resulting in one (G_{258}D; strain 0810) or two (Y_{138}D and G_{258}D; strain 1510) amino acid substitutions in the effector-binding domain of MexT. In these isolates, the sequence of *mexS*, as well as the sequences of the *mexS-mexT* and *mexT-mexE* intergenic regions, was identical to that of PA14 (Table 2). To investigate the impact of the G_{258}D substitution and the Y_{138}D plus G_{258}D substitutions on MexT activity, we complemented PA14ΔT with the *mexT* alleles from strains 0810 and 1510. The expression of the *mexE* gene, the drug resistance, and the virulence factor score of PA14ΔT were unaffected by the complementation (data not shown), indicating that neither Y_{138}D nor G_{258}D influences MexT activity, as the mutational activation of MexT would have induced *mexEF-oprN* expression in a functional MexS background. Also, these results indirectly imply that still unknown mutations are involved in the NfxC phenotype of isolates 0810 and 1510.

Impact of alterations in *mexS* on resistance and virulence. Experimental results from our university laboratory (unpublished data) and of other research groups (8) indicate that most (77% and 66%, respectively) *nfxC* mutants selected *in vitro* on MexEF-OprN substrates, such as ciprofloxacin and chloramphenicol, harbor nucleotide deletions or insertions in the *mexS* gene that are predicted to result in inactive MexS peptides. In the present study, intriguingly, only 13.6% ($n = 3/22$) of the strains turned out to carry such indels in *mexS*, whereas 45.5% ($n = 10/22$) exhibited point mutations resulting in one ($n = 9$ strains) or two ($n = 1$ strain) amino acid substitutions in the MexS oxidoreductase. The remaining 41% ($n = 9/22$) harbored a PA14-like, wild-type MexS (Table 2). The latter 9 strains were found to produce a MexT identical to that of PA14 ($n = 7$) or harbor the nonsignificant amino acid variations Y_{138}D and G_{258}D ($n = 2$; see above). As the *mexS-mexT* and *mexT-mexE* intergenic regions were 100% identical between the 9 strains and PA14, these results unambiguously demonstrate that mutations in still unknown loci (other than *mexS* and *mexT*) are able to upregulate *mexEF-oprN* expression in clinical strains.

Because amino acid substitutions may have less dramatic effects on MexS activity than disruption of the *mexS* gene, we cloned the 13 mutated *mexS* alleles in plasmid mini-CTX and complemented mutant PA14ΔS by chromosomal insertion of the cloned genes into the *attB* site. RT-qPCR experiments showed that all the transconjugants except one (complemented with MexS-V_{104}A from strain 1307) significantly overexpressed the efflux operon (Table 3). The *mexE* mRNA levels were significantly correlated (Spearman's rho = 0.96, $P < 0.01$) with the ciprofloxacin MICs (Fig. 1A). As expected, complementation with indel-carrying *mexS* alleles (from strains 1709, 1711, and 0607) failed to decrease the expression levels of *mexE* or the MICs of chloramphenicol (2,048 μg ml^{-1}) and ciprofloxacin (4 μg ml^{-1}) (compared with those for PA14ΔS; Table 3). Wild-type levels of resistance to imipenem, ticarcillin, and amikacin were also not restored in the null mutant upon complementation. Similar results were obtained with MexS-L_{263}Q (from strain 2609), supporting the notion that this mutation is strongly detrimental to MexS activity. As an indication that the remaining mutations (except the well-tolerated variation V_{104}A from strain 1307) partially compromise but do not abolish MexS activity, complementation of PA14ΔS with other MexS variants reduced the level of *mexE* expression from 1.9- to 20.3-fold and its level of resistance to both chloramphenicol and ciprofloxacin from 2- to 8-fold (Table 3). This was accompanied by the restoration of wild-type susceptibility to imipenem, ticarcillin, and amikacin in 1 (strain 2310 with MexS-F_{253}L), 8, and 0 complemented mutants, respectively. Upon complementation with MexS-F_{253}L, gene *oprD* expression was increased to

TABLE 3 Genotypes and phenotypes of PA14ΔS complemented with mutated *mexS* alleles from clinical isolates

Strain	MexS sequence (339 aa[f])	Transcript level[a]						MIC (mg ml^{-1})[b]						Virulence factor activity[c]					
		mexE	*mexS*	*mexT*	*oprD*	*mexB*	*mexY*	CHL	CIP	IMP	MEM	TIC	AMK	Biofilm formation (OD_{600})	Swarming motility	Elastase production (mm)	Hemolytic activity[d] (%)	Pyocyanin production[d] (%)	Virulence score[e]
PA14	WT[g]	1	1	1	1	1	1	64	0.12	1	0.5	16	2	**2.6**	+	**18**	**72**	**100**	5
PA14ΔS	Δ809 bp (aa 1–809)	**427**	ND[h]	0.9	0.3	0.4	0.4	2,048	4	2	1	8	0.5	0.4	−	**12**	22	13	1
$PA14\Delta S_{PA14}$	WT	1.9	1.3	1.5	1	1	1	64	0.12	1	0.5	16	2	**2.7**	+	**18**	**79**	**92**	5
$PA14\Delta S_{1307}$[i]	$V_{104}A$	0.8	1.2	1.4	1.8	1.1	1.2	64	0.12	1	0.5	16	2	**4.4**	+	**16**	**70**	**114**	5
Strains with mild substitutions in MexS																			
$PA14\Delta S_{2310}$	$F_{253}L$	**21**	4	1	0.9	1	0.8	256	0.5	1	0.5	16	1	**2.8**	+	**16**	**70**	**84**	5
$PA14\Delta S_{2505}$	$D_{44}E$	**35**	5.2	1.2	0.4	0.7	0.7	512	1	2	1	16	1	**1.7**	+	**17**	**70**	18	4
$PA14\Delta S_{3005}$	$S_{60}F$	**71**	4.8	1.2	0.4	1	0.7	512	1	2	1	16	1	**1.7**	+	**16**	**68**	18	4
$PA14\Delta S_{0911}$	$F_{185}L$	**59**	3.7	1.5	0.6	1.4	0.8	512	1	2	1	16	1	**2.2**	+	**17**	**58**	40	4
$PA14\Delta S_{1009}$	$V_{73}A + L_{270}Q$	**43**	5.8	1.4	0.5	1.2	0.8	512	1	2	1	16	1	**1.8**	+	**16**	**53**	21	4
$PA14\Delta S_{0801}$	$C_{245}G$	**83**	4	1.7	0.5	1.1	0.8	512	1	2	1	16	1	**2.3**	+	**16**	**71**	**70**	5
$PA14\Delta S_{1409}$	$A_{166}P$	**83**	5.1	1.9	0.4	1.2	0.8	1,024	2	2	1	16	1	**1.6**	+	**16**	**67**	22	4
$PA14\Delta S_{2311}$	$S_{60}P$	**230**	9.1	1.7	0.4	1	0.8	1,024	2	2	1	16	1	**1.2**	+	**16**	**59**	33	4
Strains in which MexS is inactivated																			
$PA14\Delta S_{2609}$	$L_{263}Q$	**320**	6.9	1.1	0.2	0.8	0.5	2,048	4	2	1	8	0.5	0.2	+/−	**16**	21	14	1
$PA14\Delta S_{1709}$	Δ8 bp (aa 710–718)	**334**	5.1	1.7	0.5	0.6	0.4	2,048	4	2	1	8	0.5	0.6	−	**13**	32	17	1
$PA14\Delta S_{1711}$	ΔC_{293}	**404**	2.6	1.7	0.7	0.6	0.6	2,048	4	2	1	8	0.5	0.7	−	**11**	37	13	1
$PA14\Delta S_{0607}$	Δ30 bp (aa 927–956)	**492**	8	1.9	0.5	0.8	0.6	2,048	4	2	1	8	0.5	0.6	−	**13**	20	21	1

[a] Expressed as a ratio relative to that of wild-type reference strain PA14. *nfxC* mutants (the values for which are in bold) have a *mexE* transcript level of ≥20.

[b] CHL, chloramphenicol; CIP, ciprofloxacin; IMP, imipenem; MEM, meropenem; TIC, ticarcillin; AMK, amikacin.

[c] The results for virulence factors are in bold when they are positive or considered significant. OD_{600}, OD at 600 nm.

[d] Hemolytic activity and pyocyanin production were measured on stationary-phase cultures after 18 h of growth (see Materials and Methods for details).

[e] The score was determined from the results of each test (biofilm formation, swarming motility, elastase production, hemolytic activity, pyocyanin production), positivity by each of which was given a value of 1. Of note, swarming motility and biofilm formation are correlated with rhamnolipid production.

[f] aa, amino acid.

[g] WT, wild type.

[h] ND, not detected.

[i] The strain tolerated substitutions in MexS.

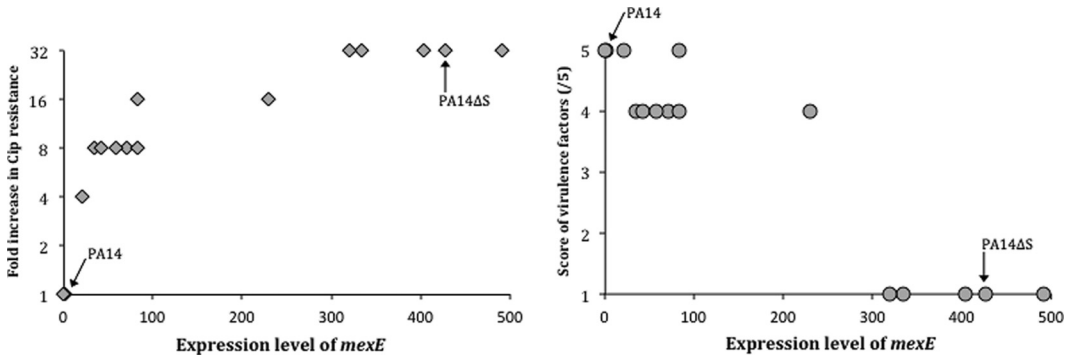

FIG 1 Correlation between levels of *mexE* expression, resistance, and virulence factor production in strain PA14ΔS complemented with *mexS* alleles from 13 clinical isolates. The expression levels of the *mexEF-oprN* operon, as assessed by RT-qPCR of the *mexE* gene, are expressed as the ratios to the level of *mexEF-oprN* expression by wild-type strain PA14 (set at a value of 1, by definition). Ciprofloxacin (Cip) MICs (diamonds) are expressed as ratios relative to the ciprofloxacin MIC for PA14. The virulence factor scores (circles) were determined by the number of positive results by 5 different tests (biofilm formation, swarming motility, elastase production, hemolytic activity, and pyocyanin production), as indicated in Table 3. The negative and positive controls, strains PA14 and PA14ΔS, respectively, are indicated. The relationships between the variables *mexE* expression and ciprofloxacin MIC (Spearman's rho = 0.96, $P < 0.01$) and the variables *mexE* expression and virulence factor scores (Spearman's rho = −0.87, $P < 0.01$) were found to be significant.

wild-type levels (0.9-fold that of PA14), while *mexE* expression was strongly repressed (20.3-fold that of PA14), providing further evidence that $F_{253}L$ only weakly affects MexS activity. The mRNA levels of the *oprD* gene in PA14ΔS were not impacted or were only marginally impacted by expression of disrupted *mexS* genes or alleles encoding amino acid variations other than $V_{104}A$ and $F_{253}L$, consistent with the unchanged resistance of PA14ΔS to imipenem upon complementation. The hypersusceptibility of *in vitro nfxC* mutants to some β-lactam antibiotics, such as ticarcillin, has been proposed to result from *nfxC*-dependent repression of the *mexAB-oprM* operon (39). As shown in Table 3, inactivation of MexS by indels or $L_{263}Q$ failed to restore wild-type ticarcillin susceptibility in transcomplemented strain PA14ΔS (for which the ticarcillin MIC was 2-fold lower than that for PA14), while less severe alterations did. Nevertheless, *mexB* expression was not significantly different among the transcomplemented mutants, with the level of expression by the mutants ranging from 0.6- to 1.4-fold that by PA14, which suggests the existence of more complex interplays between MexAB-OprM and MexEF-OprN in *nfxC* mutants, as already evoked (4). None of the *mexS* alleles except those encoding the $V_{104}A$ substitution was able to increase the levels of *mexY* expression and amikacin MICs up to wild-type levels in PA14ΔS (Table 3). However, the slight effect (a 2-fold increase in the MIC) was visible for strains with alleles with mutations resulting in mild defects but was absent for strains with alleles with mutations resulting in severe defects.

The virulence traits of the transcomplemented PA14ΔS mutants varied greatly according to the different *mexS* alleles. As for PA14ΔS, mutations leading to the complete inactivation of MexS and strong *mexE* upregulation (in alleles from strains 2609, 1709, 1711, and 0607) were associated with a low virulence score of 1 (Fig. 1B). Biofilm formation, swarming motility (Fig. 2), hemolytic activity, as well as pyocyanin production remained strongly impaired in the transcomplemented mutants (Table 3). Complementation with the other alleles (from strains 2310, 2505, 3005, 0911, 1009, 0801, 1409, and 2311) partially restored the wild-type virulence traits in PA14ΔS, yielding scores of 4 and 5. However, the level of pyocyanin production remained low in most of these complemented mutants (from 0.2- to 0.8-fold that of $PA14\Delta S_{PA14}$) and showed no evident correlation with *mexE* expression levels, as was observed in mutants $PA14\Delta S_{0801}$ and $PA14\Delta S_{1409}$ in Table 3.

Consistent with our previous conclusions, the *mexS* allele encoding the well-tolerated substitution $V_{104}A$ provided PA14ΔS with a wild-type phenotype of resistance and virulence (Fig. 2), indicating that *mexEF-oprN* overexpression in strain 1307 is due to *mexS*-independent (and also *mexT*-independent) genetic events.

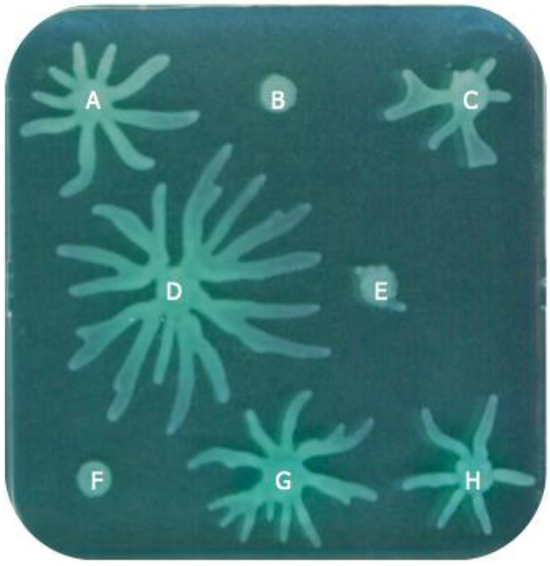

FIG 2 Swarming motility of PA14ΔS complemented with different mutated *mexS* alleles from clinical isolates. Swarming motility was evaluated as the capacity to give rise to dendrite-like patterns. The patterns for strain PA14 and $PA14\Delta S_{PA14}$ (positive controls) (A and C, respectively) and PA14ΔS (negative control) (B) are indicated. (F) Inactivation leading to an aberrant MexS protein (in $PA14\Delta S_{1711}$, for example) abolished the swarming. In most cases, substitutions in MexS, for example, $V_{104}A$ (D), $F_{185}L$ (G), and $D_{44}E$ (H), did not affect the ability of the bacteria to swarm; however, the $L_{263}Q$ substitution led to an almost complete loss of motility (E).

Other regulatory genes in *nfxC* mutants. As reported above, 10/22 clinical strains (strains 2502, 1206, 0708, 0309, 2607, 0712, 0608, 1307, 0810, and 1510) appeared to produce functional MexS and MexT proteins. Since mutations in genes coding for the global regulators MvaT and AmpR have been reported to activate the *mexEF-oprN* operon in *in vitro*-selected *nfxC* mutants (13, 14), we carried out sequencing experiments, which eventually failed to reveal alterations in these genes. Again, these results clearly indicate that other loci are implicated in pump MexEF-OprN overproduction in the clinical setting.

Conclusion. This study provides an insight into the genetic events leading to MexEF-OprN overproduction in clinical *nfxC* isolates. The hypothesis of preferential selection of partially derepressed MexEF-OprN mutants rather than fully derepressed ones *in vivo* is reinforced by our observation that *mexE* expression was lower in most clinical *nfxC* mutants (mean, 205-fold ± 187-fold that of wild-type strain PA14; median, 179-fold that of wild-type strain PA14) than in comparator strain PA14ΔS (427-fold that of wild-type strain PA14) (Table 2). None of the amino acid variations found in the *mexT* product (2/22 isolates, 9%) proved to be significant, a result consistent with the observation that LysR regulators are rarely constitutively activated by mutations (e.g., BenM in *Acinetobacter baylyi* and CysB in *Salmonella enterica* serovar Typhimurium) (40, 41). In contrast, single point mutations in the MexS oxidoreductase (9/22, 40.9%) represent a significant cause of MexEF-OprN upregulation in clinical *P. aeruginosa* strains. Consistent with these findings, a decrease in ciprofloxacin MICs from 2- to 4-fold was observed in 7 clinical *nfxC* mutants upon complementation with a plasmid-borne copy of *mexS* from strain PA14 (see Table S3 in the supplemental material). Of note, another mutation in MexS ($A_{155}V$) leading to multidrug resistance has recently been reported in a clinical isolate (42). Our results demonstrate that most MexEF-OprN-overproducing clinical strains either have a wild-type, PA14-like MexS (10/22, 45.5%) or are only partially deficient in MexS activity (8/22, 36.3%). Mutants harboring these mutations resulting in presumed mild defects display resistance and virulence traits intermediate between those of wild-type strains and strongly defective MexS mutants (4/22, 18.2%), which could account for their emergence *in vivo*. However, analysis of our clinical strains gave contrasting results (see Table S4 in the supplemental material), reinforcing the idea that the virulence of *P. aeruginosa* is multifactorial and factors other than those tested in this study may well contribute to the pathogenicity of strongly deficient *mexS* mutants, some of which were still able to cause infections. The MexEF-OprN overproducers studied here had similar growth rates (data not shown). Finally, this work indirectly demonstrates that still unknown regulators are involved in the activation of *mexEF-oprM* in 10/22 (45.5%) clinical *nfxC* mutants. We are currently trying to determine such regulatory pathways.

ACKNOWLEDGMENTS

We thank Fabrice Poncet and Amandine Mariot (SFR FED 4234, Besançon, France) for DNA sequencing data and Loïs Andrey for technical assistance. We also thank the Centre National de Référence (CNR) de la Résistance aux Antibiotiques for the provision of clinical NfxC isolates. Steffi Rocchi contributed to statistical analyses.

This work was supported by a grant from the French Ministère de l'Enseignement Supérieur et de la Recherche.

We have no competing interests to declare.

FUNDING INFORMATION

French Ministère de l'Enseignement Supérieur et de la Recherche provided funding to Charlotte Richardot.

REFERENCES

1. **Gellatly SL, Hancock RE.** 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis **67:**159–173. http://dx.doi.org/10.1111/2049-632X.12033.
2. **Poole K, Srikumar R.** 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr Top Med Chem **1:**59–71. http://dx.doi.org/10.2174/1568026013395605.
3. **Köhler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechère JC.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol **23:**345–354. http://dx.doi.org/10.1046/j.1365-2958.1997.2281594.x.
4. **Li XZ, Barre N, Poole K.** 2000. Influence of the MexA-MexB-OprM multidrug efflux system on expression of the MexC-MexD-OprJ and MexE-MexF-OprN multidrug efflux systems in *Pseudomonas aeruginosa*. J Antimicrob Chemother **46:**885–893. http://dx.doi.org/10.1093/jac/46.6.885.
5. **Schweizer HP.** 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res **2:**48–62.
6. **Köhler T, Epp SF, Curty LK, Pechère JC.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol **181:**6300–6305.
7. **Maseda H, Saito K, Nakajima A, Nakae T.** 2000. Variation of the mexT gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol Lett **192:**107–112. http://dx.doi.org/10.1111/j.1574-6968.2000.tb09367.x.
8. **Sobel ML, Hocquet D, Cao L, Plesiat P, Poole K.** 2005. Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **49:**1782–1786. http://dx.doi.org/10.1128/AAC.49.5.1782-1786.2005.
9. **Tian ZX, Fargier E, Mac Aogain M, Adams C, Wang YP, O'Gara F.** 2009. Transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*. Nucleic Acids Res **37:**7546–7559. http://dx.doi.org/10.1093/nar/gkp828.
10. **Fargier E, Mac Aogain M, Mooij MJ, Woods DF, Morrissey JP, Dobson AD, Adams C, O'Gara F.** 2012. MexT functions as a redox-responsive regulator modulating disulfide stress resistance in *Pseudomonas aeruginosa*. J Bacteriol **194:**3502–3511. http://dx.doi.org/10.1128/JB.06632-11.
11. **Frisk A, Schurr JR, Wang G, Bertucci DC, Marrero L, Hwang SH, Hassett DJ, Schurr MJ.** 2004. Transcriptome analysis of *Pseudomonas aeruginosa* after interaction with human airway epithelial cells. Infect Immun **72:**5433–5438. http://dx.doi.org/10.1128/IAI.72.9.5433-5438.2004.
12. **Llanes C, Köhler T, Patry I, Dehecq B, van Delden C, Plésiat P.** 2011. Role of the MexEF-OprN efflux system in low-level resistance of *Pseudomonas aeruginosa* to ciprofloxacin. Antimicrob Agents Chemother **55:**5676–5684. http://dx.doi.org/10.1128/AAC.00101-11.
13. **Westfall LW, Carty NL, Layland N, Kuan P, Colmer-Hamood JA, Hamood AN.** 2006. *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. FEMS Microbiol Lett **255:**247–254. http://dx.doi.org/10.1111/j.1574-6968.2005.00075.x.
14. **Balasubramanian D, Schneper L, Merighi M, Smith R, Narasimhan G, Lory S, Mathee K.** 2012. The regulatory repertoire of *Pseudomonas aeruginosa* AmpC β-lactamase regulator AmpR includes virulence genes. PLoS One **7:**e34067. http://dx.doi.org/10.1371/journal.pone.0034067.
15. **Zaoui C, Overhage J, Lons D, Zimmermann A, Musken M, Bielecki P, Pustelny C, Becker T, Nimtz M, Haussler S.** 2012. An orphan sensor kinase controls quinolone signal production via MexT in *Pseudomonas aeruginosa*. Mol Microbiol **83:**536–547. http://dx.doi.org/10.1111/j.1365-2958.2011.07947.x.
16. **Köhler T, van Delden C, Curty LK, Hamzehpour MM, Pechère JC.** 2001. Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. J Bacteriol **183:**5213–5222. http://dx.doi.org/10.1128/JB.183.18.5213-5222.2001.
17. **Olivares J, Alvarez-Ortega C, Linares JF, Rojo F, Köhler T, Martinez JL.** 2012. Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but pro-

duces specific changes in bacterial regulatory networks. Environ Microbiol **14:**1968–1981. http://dx.doi.org/10.1111/j.1462-2920.2012.02727.x.
18. **Linares JF, Lopez JA, Camafeita E, Albar JP, Rojo F, Martinez JL.** 2005. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. J Bacteriol **187:**1384–1391. http://dx.doi.org/10.1128/JB.187.4.1384-1391.2005.
19. **Lamarche MG, Deziel E.** 2011. MexEF-OprN efflux pump exports the *Pseudomonas* quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). PLoS One **6:**e24310. http://dx.doi.org/10.1371/journal.pone.0024310.
20. **Jalal S, Ciofu O, Hoiby N, Gotoh N, Wretlind B.** 2000. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother **44:**710–712. http://dx.doi.org/10.1128/AAC.44.3.710-712.2000.
21. **Llanes C, Pourcel C, Richardot C, Plésiat P, Fichant G, Cavallo JD, Merens A, GERPA Study Group.** 2013. Diversity of β-lactam resistance mechanisms in cystic fibrosis isolates of *Pseudomonas aeruginosa*: a French multicentre study. J Antimicrob Chemother **68:**1763–1771. http://dx.doi.org/10.1093/jac/dkt115.
22. **Fukuda H, Hosaka M, Hirai K, Iyobe S.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob Agents Chemother **34:**1757–1761. http://dx.doi.org/10.1128/AAC.34.9.1757.
23. **Pumbwe L, Piddock LJ.** 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **44:**2861–2864. http://dx.doi.org/10.1128/AAC.44.10.2861-2864.2000.
24. **Oh H, Stenhoff J, Jalal S, Wretlind B.** 2003. Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. Microb Drug Resist **9:**323–328. http://dx.doi.org/10.1089/107662903322762743.
25. **Ditta G, Stanfield S, Corbin D, Helinski DR.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci U S A **77:**7347–7351. http://dx.doi.org/10.1073/pnas.77.12.7347.
26. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 8th ed. M07-A8, vol 29. Clinical and Laboratory Standards Institute, Wayne, PA.
27. **Dumas JL, van Delden C, Perron K, Köhler T.** 2006. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiol Lett **254:**217–225. http://dx.doi.org/10.1111/j.1574-6968.2005.00008.x.
28. **Vasseur P, Soscia C, Voulhoux R, Filloux A.** 2007. PelC is a *Pseudomonas aeruginosa* outer membrane lipoprotein of the OMA family of proteins involved in exopolysaccharide transport. Biochimie **89:**903–915. http://dx.doi.org/10.1016/j.biochi.2007.04.002.
29. **Köhler T, Curty LK, Barja F, van Delden C, Pechère JC.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol **182:**5990–5996. http://dx.doi.org/10.1128/JB.182.21.5990-5996.2000.
30. **Ohman DE, Cryz SJ, Iglewski BH.** 1980. Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. J Bacteriol **142:**836–842.
31. **Essar DW, Eberly L, Hadero A, Crawford IP.** 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol **172:**884–900.
32. **Kaniga K, Delor I, Cornelis GR.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141. http://dx.doi.org/10.1016/0378-1119(91)90599-7.
33. **Hoang TT, Kutchma AJ, Becher A, Schweizer HP.** 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72. http://dx.doi.org/10.1006/plas.1999.1441.
34. **Tian ZX, Mac Aogain M, O'Connor HF, Fargier E, Mooij MJ, Adams C, Wang YP, O'Gara F.** 2009. MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. Microb Pathog **47:**237–241. http://dx.doi.org/10.1016/j.micpath.2009.08.003.
35. **Jin Y, Yang H, Qiao M, Jin S.** 2011. MexT regulates the type III secretion system through MexS and PtrC in *Pseudomonas aeruginosa*. J Bacteriol **193:**399–410. http://dx.doi.org/10.1128/JB.01079-10.
36. **Kumar A, Schweizer HP.** 2011. Evidence of MexT-independent overexpression of MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa* in presence of metabolic stress. PLoS One **6:**e26520. http://dx.doi.org/10.1371/journal.pone.0026520.
37. **Köhler T, Michea-Hamzehpour M, Plesiat P, Kahr AL, Pechere JC.** 1997. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **41:**2540–2543.
38. **Fournier D, Richardot C, Muller E, Robert-Nicoud M, Llanes C, Plesiat P, Jeannot K.** 2013. Complexity of resistance mechanisms to imipenem in intensive care unit strains of *Pseudomonas aeruginosa*. J Antimicrob Chemother **68:**1772–1780. http://dx.doi.org/10.1093/jac/dkt098.
39. **Maseda H, Sawada I, Saito K, Uchiyama H, Nakae T, Nomura N.** 2004. Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **48:**1320–1328. http://dx.doi.org/10.1128/AAC.48.4.1320-1328.2004.
40. **Craven SH, Ezezika OC, Haddad S, Hall RA, Momany C, Neidle EL.** 2009. Inducer responses of BenM, a LysR-type transcriptional regulator from *Acinetobacter baylyi* ADP1. Mol Microbiol **72:**881–894. http://dx.doi.org/10.1111/j.1365-2958.2009.06686.x.
41. **Colyer TE, Kredich NM.** 1996. In vitro characterization of constitutive CysB proteins from *Salmonella typhimurium*. Mol Microbiol **21:**247–256. http://dx.doi.org/10.1046/j.1365-2958.1996.6301347.x.
42. **Morita Y, Tomida J, Kawamura Y.** 2015. Efflux-mediated fluoroquinolone resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7: identification of a novel MexS variant involved in upregulation of the *mexEF-oprN* multidrug efflux operon. Front Microbiol **6:**8. http://dx.doi.org/10.3389/fmicb.2015.00008.
43. **Manoil C, Beckwith J.** 1985. TnphoA: a transposon probe for protein export signals. Proc Natl Acad Sci U S A **82:**8129–8133. http://dx.doi.org/10.1073/pnas.82.23.8129.
44. **Herrero M, de Lorenzo V, Timmis KN.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol **172:**6557–6567.
45. **Lacks S, Greenberg B.** 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. J Mol Biol **114:**153–168. http://dx.doi.org/10.1016/0022-2836(77)90289-3.